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Review

Recent advances in chromatographic purification of plasmid DNA for gene therapy and DNA vaccines: A review

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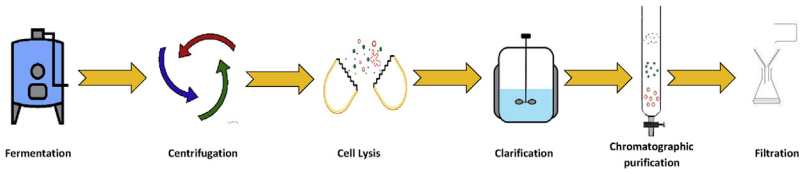
HIGHLIGHTS

- An insight on the biological, physical and chemical nature of plasmid DNA.
- Step by step in plasmid DNA production for DNA vaccines and gene therapies.
- Recent approaches and new interests in chromatographic methods for purification of Plasmid DNAs.
- Technical details and purification challenges.
- Comprehensive critical discussion of the current trends in plasmid DNA purification and future perspective.

GRAPHICAL ABSTRACT



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ABSTRACT

The wide spread of infectious diseases have provoked the scientists to develop new types of vaccines. Among the different types of vaccines, the recently discovered plasmid DNA vaccines, have gained tremendous attentions in the last few decades as a modern approach of vaccination. The scientific interest in plasmid DNA vaccines is attributed to their prominent efficacy as they trigger not only the cellular immune response but also the humoral immune responses. Moreover, pDNA vaccines are easily to be stored, shipped and produced. However, the purification of the pDNA vaccines is a crucial step in their production and administration, which is usually conducted by different chromatographic techniques. This review summarizes the most recent chromatographic purification methods provided in the literature during the last five years following our last review in 2013, including affinity chromatography, hydrophobic interaction chromatography, ion exchange chromatography, multimodal chromatography, sample displacement chromatography and miscellaneous chromatographic methods.

Contents

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1. Introduction

The discovery of vaccines has ameliorated the quality and the length of man's life. Maladies like measles and rubella turned from epidemics into well controlled in under few years of the introduction of their vaccines [1]. For instance, the period between 1964 and 1965 witnessed an epidemic spread of rubella, which infected pregnant mothers causing inborn errors and teratogenicity in their infants [2]. Thus, more than 20,000 infants were born with heart diseases, mental retardation and impaired vision and hearing [2]. Similarly, poliomyelitis [3], hepatitis A and B [4], pneumonia [5] and meningitis [6] brought about major catastrophes during their pre-vaccine era. Nevertheless, the introduction of the vaccines has remarkably declined the occurrence, morbidity and mortality of these illnesses [7].

Vaccines function by artificial induction of an immune response against infectious microbes upon introduction of the immunogens to the human body. Scholars achieved success in developing several kinds of vaccines as live attenuated vaccines, inactivated vaccines, subunit vaccines, toxoid vaccines and DNA vaccines [8]. Inactivated vaccines are prepared by application of physical or chemical means on the microbes in order to put them to death [9]. The dead microbes are injected into the human body to urge the immune system to generate antibodies against them. These vaccines cannot replicate and do not require refrigeration or special storage conditions. However, they are very weak and have to be administered more than one shot. Live attenuated vaccines are another form of vaccines, where weakened microbes act as immunogens [10]. The microbes do not cause sickness since they are enervated. Measles, mumps, and chickenpox vaccines are examples of live attenuated vaccines. However, there are dreads of mutation of the weakened microbes into contagious microbes especially with immunocompromised patients. Another downside of this kind of vaccines is that they should be preserved at low temperature, which render them strenuous to be shipped and stored [10]. Subunit vaccines made up of parts of the bacterial cell components as cell wall polysaccharides. Nevertheless, weak immune response towards these vaccines, limits their application [10].

DNA vaccination is the use of DNA particles of pathogens to induce the immunity against certain contagions. It is a novel sort of vaccination methods [11,12], which was identified as cellular and humoral immune responses stimulant. The new vaccination method became available for clinical trials after just ten years of its debut [13], and some of DNA vaccines are now available in the market like West Nile virus vaccine which is used for veterinary applications [14]. On the other hand, plasmid DNA (pDNA) vaccines are a subtype of DNA vaccines, where the plasmid encoding certain protein antigen was extracted from the pathogen cell and transformed into *Escherichia coli* (*E.coli*) [15]. The plasmids replicate in the *E.coli* cells in a process called "Up-Stream process". After that, a process of downstream is applied to obtain a purified amount of plasmids (discussed later) [16]. The purified plasmids are injected into the human body and reach the nucleus of human cells by endosomal trafficking. The transgene (plasmids) utilizes the cellular transcription machinery to produce the antigen protein, and this process could remain active for several months. Then, the synthesized protein activates the immune response through MHC-I and MHC-II pathways [1].

pDNA vaccines showed significant advantages over the traditional protein and virus-based vaccines. The production of pDNA only requires a reliable fermentation process and does not need chicken eggs or other time consuming techniques, which make its production easier and quicker [15]. Moreover, the stability of pDNA eases its storage and shipping. The most important advantage of utilizing pDNA vaccine is its ability to induce not only the antibodies but also the cell mediated components of the immune system [17]. Thus, pDNA vaccines have paid the attention of numerous investigators in the last few decades. The number of the published patents about DNA vaccines was increased from 1 in 1990 to 975 in 2017, according to Scifinder as shown in Fig. 1. Nevertheless, the immunogenicity of the pDNA vaccines is still a matter of study [15]. The advantages and the disadvantages of pDNA vaccine are listed in Table 1 [15].

In another approach, plasmid DNA also can be used in gene therapy. Gene therapy is the utilization of genes as treatment. The process comprises the transfer of a particular gene into living cells aiming at fixing an improper gene copy or to insert a new gene to treat or improve certain disease. The applications of this technique include treatment of genetic defects, cancer and viral diseases [15,18]. Many countries permitted the clinical trials of gene therapy including the United States, Europe and Australia. The first gene therapy success story was in 1990s, when scientists could treat a type of immune deficiency called adenosine deaminase deficiency [19].

In 2013, our research team provided a literature review covering recent chromatographic procedures for purification of pDNA vaccines. Here we present an extension covering the novel chromatographic methods issued between the period of 2013–2017 including; affinity chromatography [20], anion exchange chromatography [21], multimodal chromatography [22] hydrophobic-interaction chromatography [15] and size exclusion chromatography [23]. In this article, we will discuss and review the most recent chromatographic methods for purification of pDNA that was accomplished in the last five years.

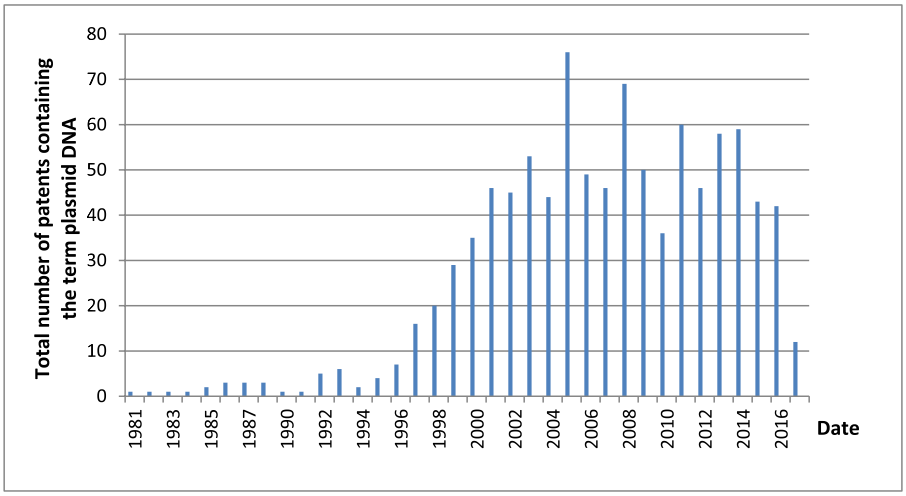

Fig. 1. Number of patents in the field of pDNA vaccines.

Table 1
The advantages and disadvantages of pDNA vaccines.

Disadvantages	Advantages
Low immunogenicity in humans and large animals	Cheap in comparison to other protein or micro-organism based vaccines
Low DNA cellular uptake in high body masses	The immune response is long and no need for booster shots
The long immune response may cause inflammation or production of autoantibodies	Stimulates higher immune response than the other vaccines
The immune response is limited to the protiens and not to non-protein based antigens like polysaccharides	High stability and easier in shipping and storage
Potentiality to alter the cell growth control genes	Low infection risk
Simply developed and produced.	

2. Plasmid DNA

Plasmids are self-replicated, tiny, and circular double stranded DNA molecules detached from the DNA of eukaryotic and prokaryotic organisms' cells. A plasmid can carry certain genes that are essential for the survival of the organism, like antibiotic resistance genes and genes responsible for synthesis of certain proteins [24]. The plasmids have the ability to transmit from one cell to another by three mechanisms; transformation, transduction and conjugation. The size of plasmids is measured by Kilo base unit (Kbp), while its size can range from 1 to 200 Kbp [24].

The pDNA has five conformations which are different in size and speed in electrophoresis, namely; open-circular (oc) pDNA, relaxed circular pDNA, linear pDNA, supercoiled (sc) pDNA and supercoiled denatured pDNA [24]. The oc pDNA has one strand and circular in shape. The relaxed circular pDNA is double stranded but relaxed due to an enzymatic effect. The linear pDNA is cut double stranded pDNA which have free ends. Sc pDNA is a dense conformation of pDNA consists of complete double strand with the normal twist and shape. The sc denatured pDNA is similar to sc pDNA but less dense because of the presence of unpaired regions in the pDNA helix [24]. The five isoforms are different in charge, size, hydrophobicity and base exposure. The sc isoform is the one of choice in DNA vaccination and gene therapy due to its outstanding stability and eminent antigenicity [25]. The supercoiling of the sc isoform increases its base exposure and its charge more than the other isoforms [26]. Researchers took advantage of this characteristic in separation of sc pDNA isoform (discussed later). However, the sc isoform may convert into oc or linear isoform under certain physical and chemical procedures, which may affect its purification yield and recovery [27].

3. Plasmid manufacturing

3.1. General idea

Production procedures of pDNA must be set upped before market approval by developing well-studied manufacturing processes. Improvement of the production process by further investigations must be taken into account during the production [28]. The production process of pDNAs involves a series of safe and effective procedures (Fig. 2) that are established and performed for regular manufacturing of a certain quantity of pDNA. Firstly, cell banks of the intended pDNA are prepared, as well as the starting materials are chosen and analysed. After that, the units of cell culture and downstream processing are specified, sorted, designed, and functioned to create a bulk of pDNA [28]. Eventually, final processes must then be implemented to make the refined pDNA suitable for its definitive form by taking into account certain aspects, like the route of administration, the dosage form, the additives, the recommended dose, packaging and storage. After this stage, the product becomes available for pharmaceutical marketing and clinical trials [28].

3.2. Cell culture

After selecting a specific strain with the target plasmid, cloning is implemented into *E.coli* cell. This is followed by the promotion of pDNA replication by fermentation held to provide a cell bank of the plasmid containing cells [29]. Certain measures are taken to minimize the probability of recombination and degradation of pDNA. The growth conditions of the cells should be carefully selected and optimized; such as the growth media, bioreactor operating

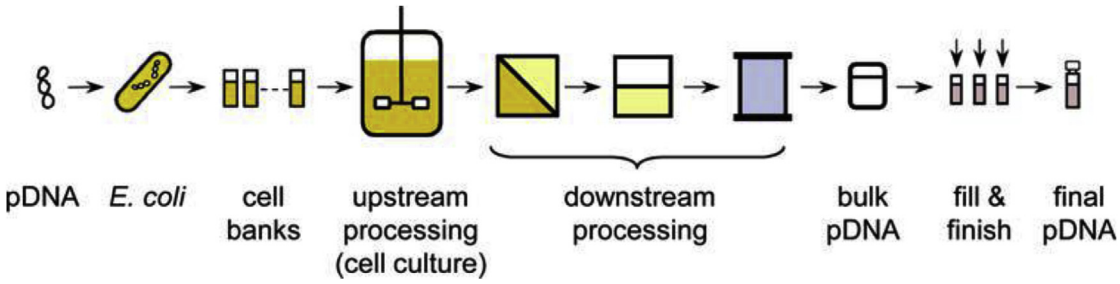

Fig. 2. General idea about the plasmid DNA production steps [28].

parameters and cultivation technique, to get the most out of pDNA yield. At its best conditions, operation may yield up to 2.0 g/L [30].

3.3. Downstream processing

The main aim of the downstream process (Fig. 3) is to obtain the pDNA in a reasonable purity that meets the pharmaceutical specifications, and get rid of the other cell impurities like RNA, proteins and genomic DNA [15]. The whole process could consist of three stages namely preliminary purification, intermediate purification and final purification. In the preliminary purification step, the high density broth of *E.coli* cells harvested from fermentation step was collected using different techniques like centrifugation or microfiltration. Subsequently, the cells are lysed to liberate the pDNA by means of lysis techniques, like alkaline lysis or thermal lysis, to take out the lysates. In the second step, clarification and pre-purification procedures are taken to decrease the levels of impurities in the lysates and prepare the pDNA for the final purification stage [15]. The pre-purification step can be performed by means of ultrafiltration [31], tangential flow filtration [23], precipitation [32], adsorption [33] or aqueous two-phase systems [34]. The pre-purification step is critical for the production of pDNA vaccines, but it is not enough to obtain the pDNA vaccine in the desired purity. Thus, a final step must be implemented to compile to the purity stated by the pharmaceutical regulatory bodies.

3.3.1. Chromatographic purification of pDNA

The current progresses in biotechnology have allowed the investigators to explore and produce complex biomolecules like pDNA through the application of novel techniques, which might have the ability to improve the human health care. The purification step is the assessment standard of the entire production process worldwide because of its importance and cost [25]. Various purification procedures can be taken like precipitation [35], ultrafiltration [31] and aqueous two-phase systems [36]; nevertheless, liquid chromatography is certainly the most extensively implemented technique [37]. The complex content of the plasmid containing lysates must be taken into consideration when exploring a purification method of pDNA, because other cell constituents (like RNA, gDNA and proteins) are also present in the lysate media [38]. The purification technique must guarantee at least 97% purity of the final obtained pDNA, with at least 80% sc pDNA, to comply with the requirements of the regulatory agencies. According to the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA), the pDNA vaccines should not contain more than 1% of gDNA, host proteins, RNA and endotoxins [38,39]. Thus, the demand for an efficient pDNA purification tool is crucial for the manufacturing of pDNA based vaccines.

Therefore, many chromatographic methods are utilized solely or mixed as purification techniques of pDNA [15]. The chromatographic purification step usually faces plentiful challenges due to the physiochemical properties resemblances, like size, hydrophobicity and charge, between pDNA and the other lysate components, which may complicate the separation process [38]. Accordingly, the purification of pDNA may require more than a single chromatographic step to obtain the desired pDNA purity [23]. Moreover, the purification of pDNA by chromatography may be confronted with other encounters, like the limited number of the existing stationary phases that have the capacity and ability to bind to the large biomolecules like pDNA [38,40]. Furthermore, soft beads packed columns may be susceptible to wricking as well as mass transfer difficulties. These limitations led the researchers to evolve monolith column for industrial use [41].

Monolith is a single piece of porous solid of notable efficient continuous homogenous stationary phase layer, which allows high flow rates and high mass transfer with low back pressure [40]. These properties nominate monolith to be an efficient and rapid purification and analysis tool of large macromolecules such as pDNA [42,43]. Furthermore, additional supports were developed to get rid of the diffusion limitation and enhance the dynamic binding capacity such as absorbents like porous glass beads [44]. Thus, liquid chromatography is the method of choice for purification of pDNA in large scales due to its superb capacity, recovery and

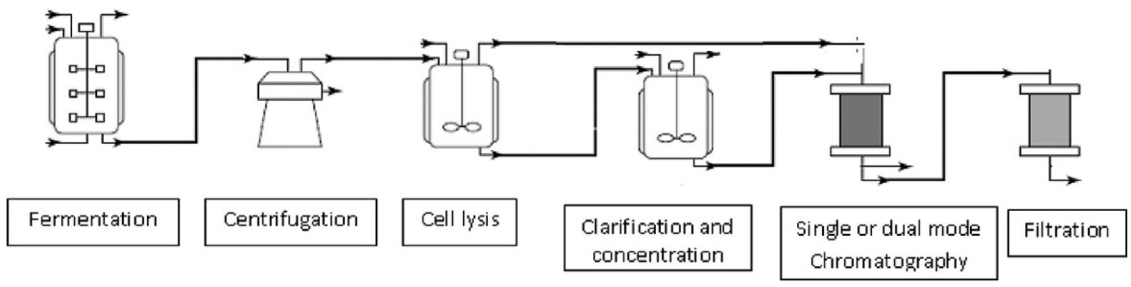

Fig. 3. Strategies for purifying supercoiled plasmid DNA. Pure supercoiled plasmid DNA can be obtained by moving directly from cell lysis to chromatography or by performing clarification and concentration before chromatography [15].

versatility [15]. However, the main challenge facing the scholars is to optimize old chromatographic methods or to acquire new ones, to separate pDNA in good yield and great purity. Selection of the type of the chromatographic procedures employed depends on the nature of the pDNA as well as the other lysate components [25].

3.4. Affinity chromatography

Affinity chromatography is a form of chromatography, usually used for purification of biological mixes depending on highly selective reversible interactions between a ligand loaded onto a support and a substrate [45]. In general, the more interaction sites between the ligand and the substrate are, the higher dynamic binding capacity of the ligand will be, which might contribute to the whole technique's selectivity, specificity and efficacy. The outstanding selectivity and versatility of affinity chromatography, nominate it to be a meaningful tool for purification of several biological molecules like, pDNA, RNA, DNA and proteins [46]. The use of affinity chromatography as a selective tool for purification of pDNA from lysates and separate their isoforms (sc and oc) enticed many researchers in the last few years. Purification of pDNA from lysates is based on the fact that the pDNA bind to affinity ligands through different styles of bonds and interactions like hydrogen bonds, ionic bonds, hydrophobic interactions, etc. While the other lysate components (impurities), like RNA, gDNA, proteins and endotoxins, exert different affinities to the ligands at certain mobile phase's salt concentration, pH and temperature. Therefore, the separation of the pDNA isoforms happens as the impurities were washed out while the pDNA molecules were retained or vice versa [47,48]. Beyond that, affinity chromatography is not only used for separation of the pDNA molecules from lysates, but also it effectively isolates the sc and the oc isoforms of pDNAs. The separation between the oc and sc isoforms might be related to the difference in the conformational structures of the two isoforms. The supercoiling of the sc pDNA contributes to a superior torsional strain of this isoform, which raises the charge and the base exposure of this isoform over that of the oc pDNA isoform [26]. These differences between the sc and the oc isoforms allow the sc isoform to attach to the ligands with a different affinity than the oc isoform. By passing an elution system of changeable ionic strengths, separation between the two isoforms could take place [26].

For instance, in 2012, berenil (an anti-trypanosomal agent used in veterinary) was immobilized onto Sepharose column for the separation of pDNA samples of different sizes [pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp)], from clarified and non-clarified *E.coli* alkaline lysates [49]. Berenil undergoes hydrophobic interaction with pDNA owing to its phenyl group, and other specific interactions which might explain its high affinity to pDNA. Clarification of the lysate was preceded by precipitation with 2 M ammonium sulphate. The purification trials were implemented firstly on the clarified lysate. After the retention of all lysate components with 1.3 M of ammonium sulphate in 10 mM Tris-HCl buffer (pH 8.0), pDNA was eluted by descending the salt concentration to 0.55 M. The impurities were collected at the last stage of the previously mentioned elution process. The obtained results showed satisfactory purity of the separated pDNAs (100%) [49]; however, the environmental hazards and the economical aspect of the elevated concentration of ammonium sulphate, used in the mobile phase, are the drawbacks of this approach. Moreover, the recovery depends on the size of the pDNA, since the recovery of the small size pVAX1-LacZ was found to be 85%, which is very similar to a previous affinity chromatography method [50] (84%) and better yield than a previous HIC method [51]. Nevertheless, the recovery of the larger size pCAMBIA-1303 is very low (45%). The investigators explicated that the low recovery of the large size molecule might be ascribed to the liability of the large pDNA molecules during production processes. In order to ease the process and avoid the hazard of ammonium sulphate used in the clarification step, trials were implemented on non-clarified *E.Coli* lysate through two runs following similar chromatographic conditions. The purity of the gathered pDNA is quite sufficient, and most of the RNA was removed; yet, the obtained yield was surprisingly low (33%). Caramelo-Nunes et al.'s research could not offer a method for separation between the oc and sc pDNA isoforms [49]. In the same year, the validation of phenyl-boronic ligands derivatized onto porous glass beads as a pDNA purification stationary phase was demonstrated. The employed elution system was Milli-Q water then 1.5 M Tris (pH 8.5). Phenyl-boronic column effectively purified pVAX-GFP (3.7 kbp) and pVAX1-LacZ (6.1 kbp) with yields of 95.7% and 94.2% respectively, with reasonable purity [44]. Moreover, the researchers avoided using ammonium sulphate, which renders the method environmentally clean. The method offered clean, quick, facile, reliable and reproducible purification process; however, the scholars did not expose to pDNA molecules larger than pVAX1-LacZ [44]. To continue their trials on pDNA purification, Caramelo-Nunes et al. presented a novel method for purification of the sc pVAX1-LacZ isoform from the previously separated mixture of pDNA isoforms (oc+sc) from bacterial lysate, using Sepharose support immobilized with a small DNA ligand: intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) [52]. The team carried out several trials to conclude that DAPP-Sepharose has a great affinity to the sc isoform of pVAX1-LacZ. The best purification results were obtained by employing an elution system of gradient ascending of sodium chloride concentration (from 0.22 M to 0.55 M) or descending ammonium sulphate concentration from (2.1 M–0 M), in 10 mM acetate buffer pH 5, on Sepharose-DAPP stationary phase. However, the team favoured the utilization of NaCl in the elution system to prevent the unfavourable drawbacks of ammonium sulphate. The chromatographic separation diagram is given in Fig. 4. The separated sc isoforms showed superb purity, and the separation is pH dependent. The use of a low amount of sodium chloride in the mobile phase keeps the method clean and economic [52]. The DAPP-Sepharose system was further applied for purification of sc pDNA with different sizes from clarified E. coli lysates using low amounts of salt in the eluent and providing a high quality sc pDNA in a single chromatographic step [53].

In another approach, a recent article by Soares et al. [54] combined the previously approved selectivity of arginine ligand to pDNA [46] with the well-proven eminent mass transfer properties and superior binding capacity of monolith support [40] for the isolation of two sc pDNAs of different sizes; sc pHPV-16 E6/E7 (8.702 kbp) and sc pVAX1-LacZ (6.05 kbp), from clarified lysate. The elution system is based on the employed pre-purification techniques. In case of pre-purification by Qiagen Kit, the elution system is two steps; the first one is 560 mM NaCl in 10 mMTris–HCl and 10 mM EDTA buffer (pH 8.0), to remove the lysate impurities, then 2 M NaCl at the same buffer to elute the bound sc isoforms. In case of pre-purification by isopropanol/ammonium sulphate/PD10, the elution system is ascending concentrations of NaCl from 600 mM in10 mM Tris–HCl/10 mM EDTA buffer (pH 8.0) to 740 mM then to 1 M [54].

The arginine monolith binding capacity was compared to that of arginine-agarose to evaluate the monolith as a support, and the findings proved that arginine monolith possesses superior binding capacity as shown in Fig. 5 [54]. The use of low concentrations of salt is an economic advantage of this method. In addition to, the precluding of ammonium sulphate in the mobile phase, keeps the method environmentally clean. The purified larger size sc pHPV-16 E6/E7 demonstrated high purity (>99%); nevertheless, the yield is extremely low (39%). In contrast, the yield of the smaller sc pVAX1-

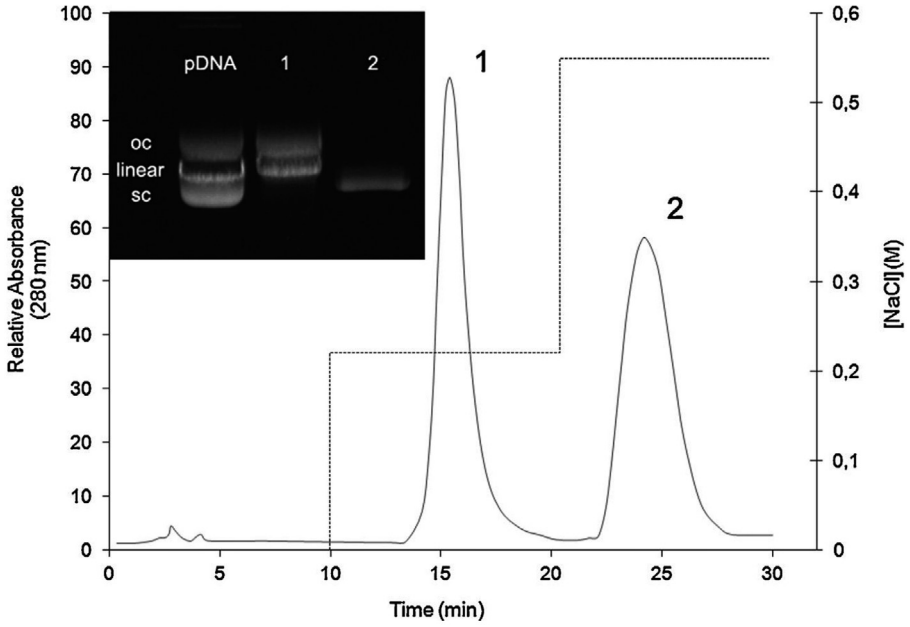

Fig. 4. Chromatographic separation of pDNA (25 _L, 600 _g/mL) isoforms on DAPP-Sepharose and agarose gel electrophoresis of the peak fractions. Lane pDNA: sample of pDNA injected onto the column (mixture of oc and sc isoforms, plus the linear isoform obtained from the enzymatic digestion of the plasmid with Hind III). Lane 1: oc and linear pDNA fraction collected from peak 1 using as eluent acetate buffer 10 mM pH 5 with 0.22 M NaCl. Lane 2: sc pDNA fraction collected from peak 2 using acetate buffer 10 mM pH 5 with 0.55 M NaCl as eluent [52].

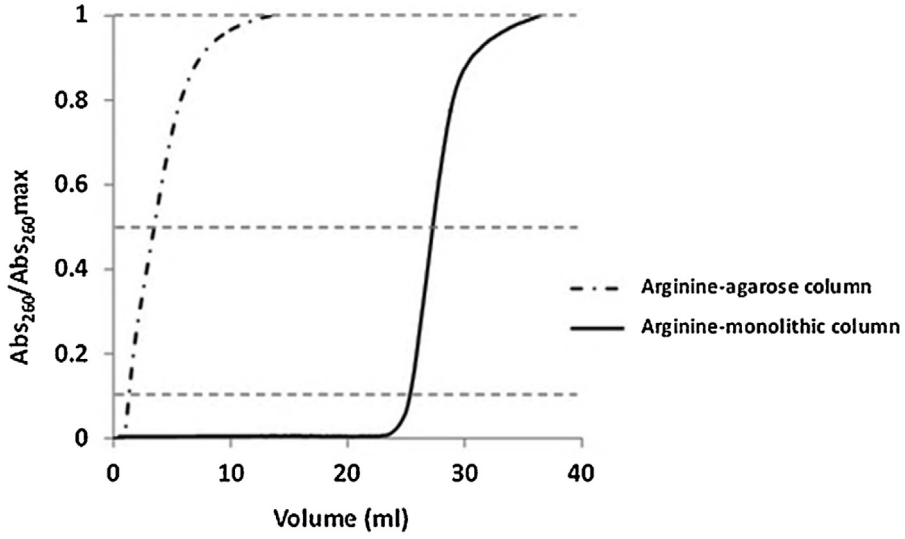

Fig. 5. Breakthrough experiments with arginine monolith and conventional arginine-agarose matrix. Flow rate: 1 mL/min; feedstock: HPV-16 E6/E7 plasmid solution pre-purified with Qiagen kit (0.05 mg/mL) was homogenized in 10 mM Tris–HCl and 10 mM EDTA buffer pH 8.0 [54].

LacZ was higher (86%) with similar purity, which exculpates the method procedures from the low yield of sc pHPV-16 E6/E7. Thus, the low yield of sc pHPV-16 E6/E7 might be related to the molecule properties itself, which might keep it unstable in the production process [54].

With the intention of developing cost and time effective and minimal salt consumable method, Caramelo-Nunes et al. utilized Berenil-Sepharose column yet again for the separation of pVAX1-LacZ from clarified lysate [55]. A concentration of 0.55 M of ammonium sulphate in 10 mM Tris-HCl buffer at pH8.0 was employed at first to elute the pDNA then the salt concentration was lessened to 0 M to elute the retained impurities. The low ammonium sulphate concentration used decreases the cost and the ecological dangers of this method in contrast to their previous investigations [49,51]. The purified pDNA was collected with 87% recovery and more than 99% purity. Moreover, the two-step elution speeds up the purification process of pDNA and saves time and efforts. However, the method did not prove feasibility in separating the sc pDNA isoform from the oc/sc mixture [55]. Another study suggests that methionine could interact with pDNA by strong hydrophobic and weak thiophilic interactions which may explain its great binding capacity with pDNA [56]. Immobilization of L-methionine onto agarose support was implemented to purify three sc isoforms of pDNAs of different sizes; HPV-16 E6/E7 plasmid,

pUC19 and p53-encoding pDNA, from clarified lysates. Numerous trials were exerted to optimize the elution conditions by changing the temperature, pH and salt concentration. The best separation was achieved at 5 °C by lowering the ammonium sulphate concentration from 2.35 to 1.7 M gradually at 10 mM Tris-HCl, at pH 8 buffer, then using the buffer alone for the elution of the bound sc pDNA [56]. The purified sc pDNA were pure enough; nonetheless, the recoveries of p53-encoding plasmid, pUC-19, and HPV-16 E6/E7 were very low, 39%, 20% and 30%, respectively. The authors referred the low recoveries to the plasmid characteristics not to the method without giving a critical explanation. Furthermore, the method uses considerably high concentrations of the costly and non-ecofriendly ammonium sulphate which are the main drawbacks of this method [56].

A study on the behavior of nongrafted carbonyldiimidazole monolithic disks in separation of sc pDNAs of different sizes was operated by Bicho's team [57]. The team employed their trials on three different sizes of pDNAs; pcDNA3-based plasmid (14 kbp), pEGIP (10.292 kbp) and pUC19 (2.686 kbp) in one chromatographic step using purified samples of sc and oc isoforms [57]. It was concluded that, the concentration of ammonium sulphate in the elution system and the dynamic binding capacity of the column are inversely proportional to the size of the pDNA as shown in Fig. 6; since, the larger pDNAs are, the higher their base exposure and the higher interactions with the stationary system. On the other hand, the dynamic binding capacity was found to be directly proportional to ammonium sulphate concentration at low flow rates and low pH (Fig. 6) [57]. This carbonyldiimidazole monolithic disk was also applied to purify pDNA suitable for pharmaceutical applications with high purity level and satisfactory recovery [58]; however, the elevated concentration of ammonium sulphate used throughout this method, is not ecologically preferable. Furthermore, a group of Chinese researchers undertook an investigation to explore the effect of spacer arm and ligand density of arginine-based beads on the isolation of a small pDNA molecule; plasmid pET-28a (5.369 kbp) [59]. Elution was done using 10 mmoL/L Tris-HCl buffer at pH 8 (buffer A) at first, then 1.0 mol/L NaCl was added to buffer A (buffer B) to elute the bound pDNA. Another chromatographic procedure

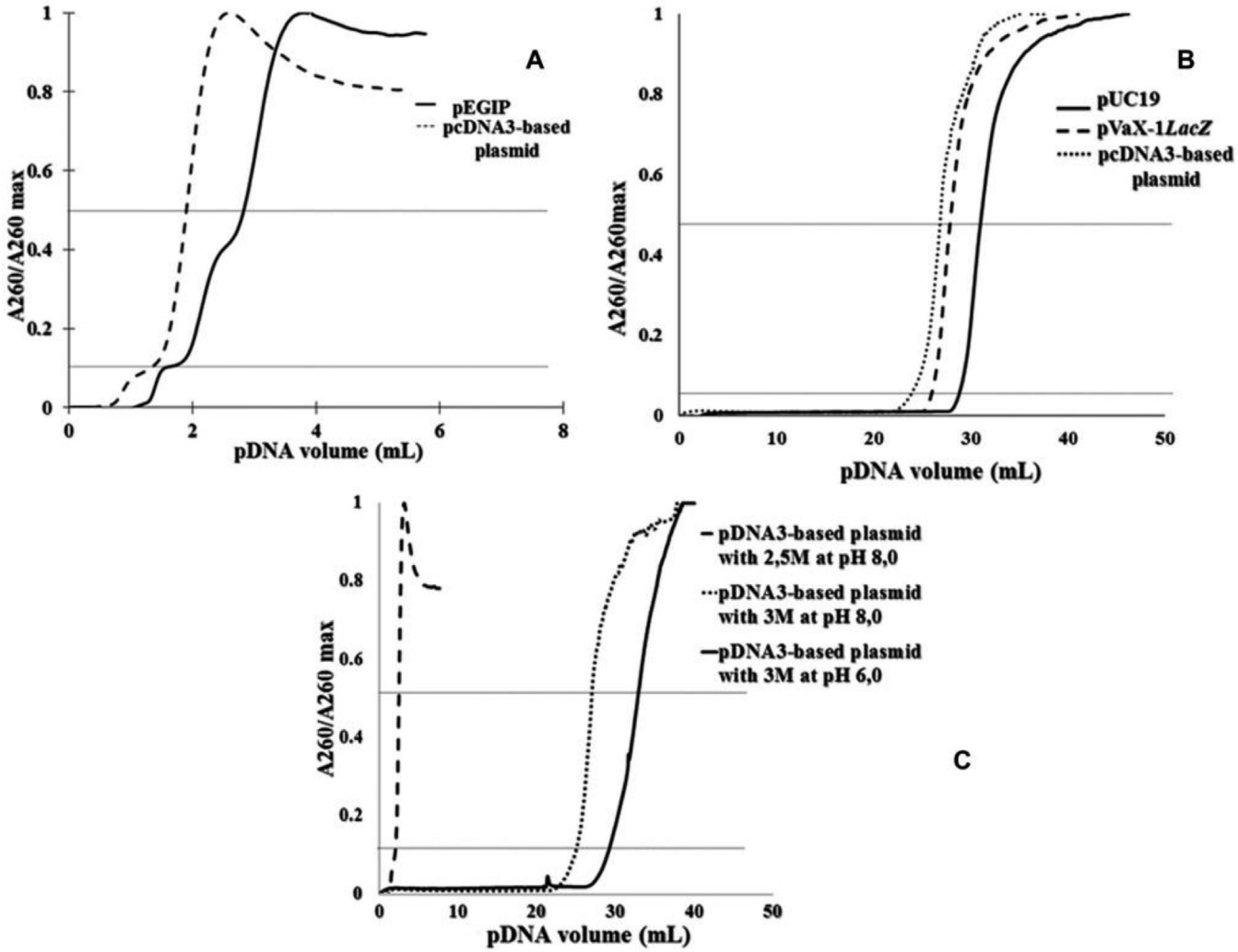

Fig. 6. Breakthrough curves of sc pDNA (0.05 mg/mL) in CDI disk at room temperature using 1 mL/min flow rate. (A) Influence of large plasmids in the DBC, pEGIP (10.292 kbp), and pcDNA3-based plasmid (14 kbp), with 2.5 M (NH4)2SO4. (B) Plasmid size effect on the DBC, pUC19 (2.686 kbp) pVAX-1LacZ (6.05 kbp), and pcDNA3-based plasmid (14 kbp) using 3 M of (NH4)2SO4 concentration. (C) Effect of salt concentration (2.5 and 3 M of (NH4)2SO4) and pH (8.0 and 6.0) in the DBC of pcDNA3-based plasmid (14 kbp) [57].

was followed to separate the sc-pDNA using ascending concentration of buffer B employing the same stationary phase. Clarified samples were injected directly, which eases the process and shorten the time that may be made in the pre-purification processes. The presence of electrostatic interaction and hydrogen bonds between pDNA and the ligand is the reason behind the great selectivity of arginine [59]. The researchers found that ligand density and spacer arm are directly proportional to the dynamic binding capacity, and after trials, the ligand density of the column of 47 mmoL/L might contribute to the best recovery. The purified sc pDNA showed reasonable purity 93.8% and good yield (over 80%) at 3 mL loading dose [59]. Generally, the research provides eco-friendly, cheap, rapid and effective procedures, however, the purification of higher molecular weight sc pDNAs following these procedures was not investigated [59]. Once again, another group of investigators [60] exploited the advantages of monolith as a support, to combine it with histamine as a trial for improving previous multimodal chromatographic procedures [61]. The method was designed to separate large volume of the sc isoform of pVAX1-LacZ from clarified *E.coli* lysate (86 mL), using ascending NaCl or descending ammonium sulphate as mobile phases (simple strategy) or by combining the two procedures (combined strategy). The simple strategy presented better results in terms of selectivity and purity; however, the combined strategy might be beneficial in case of column overloading. The best recovery (97%) and purity (99%) of sc pDNA was secured through the elution system of ascending concentration of NaCl; 1.70 M, 2.08 M then 3 M NaCl in 50 mM citrate buffer (pH 5.0) [60]. Fortunately, this elution system gave better results than descending ammonium sulphate. The researchers offered preparative procedures for purification of large volumes of sc pDNA. Moreover, the neatness, simplicity and efficiency of the method qualify it to be an excellent approach for preparation of pure sc pVAX1-LacZ. Nevertheless, the isolation of larger sc pDNA molecules was not explored yet [60].

On a similar approach, the same research team carried out another investigation to explore the mechanisms of histamine and agmatine ligands, immobilized onto monolithic disks, in separation of pVAX1-Lacz sc isoform [62]. Ascending gradient of NaCl in acidic pH, and descending gradient of ammonium sulphate in alkaline pH were intended for use as mobile phases. Both elution systems successfully separated pDNA isoforms; nevertheless the researchers preferred the application of NaCl system to avoid the environmental hazards of ammonium sulphate. It was proven that changing in the mobile phase and the pH, changes the interactions between pDNA with histamine or agmatine. The research group also proved that, the stability of different sc pDNA molecules of different sizes: 2.69-kbp pUC19, 6.05-kbp pVAX1-LacZ and 14-kbp pcDNA3-myc-FLNa S2152A, might be related to the elution system's ionic strength and pH [62]. Additionally, in order to explore the versatility of dipeptides as ligands, two dipeptides: L-tyrosine-L-tyrosine and L-tyrosine-L-arginine, were synthesized and coupled to epoxide-activated Sepharose support at basic pH and 70 °C [32]. Gradient descending ammonium sulphate concentration in 10 mM Tris-HCl and 100 mM of HEPES was the mobile phase at different temperatures (5 °C, 10 °C and 25 °C) for separation of sc pVAX1-LacZ and sc pcDNA3-FLAG-p53 isoforms [32]. It was demonstrated that the selectivity and the retention of the sc pVAX1-LacZ are lower than that of sc pcDNA3-FLAG-p53, although both are almost of the same size. This difference might be related to the dissimilarities in the DNA sequences and the structural configurations of the two sc pDNA isoforms. The temperature was found to be inversely proportional to the dynamic binding capacity, as the pDNA molecules demoed higher binding affinity to the dipeptides ligands with low temperature and vice versa. The research procedures proved the possibility of purification of pDNA using L-tyrosine based-Sepharose; however, the yield and the recovery were not mentioned. Moreover, the use of ammonium sulphate as elution system is a controversial [32].

In view of Soares et al. research [54] in 2013, Almeida et al. implemented a novel trial to improve the recovery of sc HPV-16 E6/E7 (8.702 kbp) plasmid-based vaccine by optimizing the conditions of arginine monolith column [26]. The study was based on Composite Central Face through optimization of three factors; the binding concentration, washing concentration and the pH of the mobile phase. The elution took place through three steps. The first step is the equilibration step, where NaCl concentration was between 500 nM and 600 nM in Tris-HCl/EDTA buffer. Then the washing step, where the salt concentration was raised to between 650 and 750 nM to wash RNA, oc pDNA and other impurities. At the last step, the salt concentration reached 1 M at the same buffer in order to liberate the sc pDNA [26]. The pH ranges from, 7.5 to 8. Low pH increases the pDNA retention since pKa of arginine is 12. Hence, at low pH, arginine carries positive charges which interact with sc pDNA. The recovery was successfully improved from 39% to 83.5% with 100% purity. The method is eco-friendly and can be considered as a promising approach for the purification of large sc pDNA molecules. Furthermore, the interference of Composite Central Face technique in designation of chromatographic purification method of pDNA, is a promising approach which might be useful in optimizing the pDNA purification in the future [26]. In 2015, the attractive properties of monolith provoked Amorim et al. to immobilize L-histidine and its derivatives onto it, for purification of three different sized pDNAs (pVAX1-LacZ, HPV-16 E6/E7, and pcDNA3) [63]. The separation occurred by decreasing the concentration of ammonium sulphate in the elution system [63]. However, lowering the ammonium sulphate concentration was needed for separation of sc pDNAs in the 1-benzyl-L-histidine monolith, due to the high interactions evolved. The researchers compared the binding capacity of L-histidine monolith with that of L-histidine agarose, to find the first one's is higher by 29 times. The adequate binding affinity of pDNA to the ligands, especially with HPV16 E6/E7, allowed the investigation team to purify sc isoforms. However, the method is solely a screening method, with the authors not mentioning the yields or the recoveries of the separated sc pDNA molecules [63].

On a similar approach, the successful results of amino acid ligands motivated Santos and their co-workers to explore L-tryptophan as a ligand [64]. The presence of aromatic ring side chain and the rigid indole ring in L-tryptophan, contributes to its interactions with the biomolecules like pDNA [65,66]. Based on this hypothesis, L-tryptophan and its dipeptide derivatives (L-tryptophan-L-tryptophan and L-tryptophan-L-arginine) were investigated as affinity ligands immobilized onto Sepharose CL-6B to separate sc pVAX-LacZ and sc pPH600 from their oc isoforms. The separation took place with decreasing in the concentration of ammonium sulphate from 2.65 M to 0 M in 100 mM HEPES acid (pH7.4) at 10 °C. The researchers explained that the interaction between the ligands and the pDNA depends on the hydrophobic interaction and π-π stacking of 5-mononucleotides of pDNA with the L-tryptophan based ligands. The investigation team successfully proved the purification efficiency of the L-tryptophan-Sepharose; this paves the way for further investigations to implement these procedures directly on the *E.coli* lysate [64]. In a parallel approach, naphthalene tripodal was immobilized onto epoxy-activated Sepharose CL-6B, for the first time, to isolate sc pVAX1-*LacZ* [20]. The interaction between the new ligand and the 5′aminonucleotides was found to be hydrophobic and π-π stacking. Gradient increase in sodium chloride concentration of the elution system from 95 mM to 550 mM at Tris-HCl pH 8 at 4 °C achieved the best separation. The presence of the three amino acids in naphthalene tripodal promotes its interactions

with pDNA which results in better affinity than DAPP upon comparison. Overall, the investigators provided a quick (two steps), clean and low salt consumable method that can be used for purification of sc pVAX1-*LacZ* [20].

To conclude, Affinity chromatography is a facile purification tool, which ascertained its feasibility in separation of pDNA. The versatility of the ligands eases the task of the investigators to find robust and selective pDNA isolation methods. The affinity chromatography-isolated pDNAs demoed sufficient purity and appropriate recovery with small pDNA. However, incorporation of ammonium sulphate in the elution systems might have harmful environmental impacts. The researchers could develop novel methods with low ammonium sulphate concentrations if needed. Moreover, purification of the large size pDNA molecules by affinity chromatography was not achieved in reasonable yields due to the vigorous production and extraction procedures. Further investigation might be needed to improve the yield upon purification of large pDNA molecules.

3.5. Hydrophobic interaction chromatography (HIC)

Due to the differences in the hydrophobicity of the components of the bacterial lysates, HIC gained a great attention as a purification tool of pDNA and pDNA isoforms [67]. Separation of pDNA by HIC depends on the concentration of the salts in the mobile phase buffer passing through a hydrophobic support. Thus, high salt concentration enhances the interaction between the pDNA and the hydrophobic support and vice versa [43]. HIC separations are performed in four steps; equilibration, sample application, elution and regeneration [68]. Equilibration step is to prepare the stationary phase for separation by passing a buffer containing salt (usually sodium phosphate or ammonium sulphate). Sample application and wash step is put in place to introduce the sample into the stationary phase and wash the weakly bound impurities by moderate concentration of the salt. Elution stage is carried out by gradient decrease of the salt concentration to separate the pDNA isoforms. The final step is the regeneration of the stationary phase, to remove all the strongly bound molecules [68]. The conditions of HIC are mild and “protein friendly” giving the advantage of the excellent recoveries [69].

In 2013, Bonturi et al. tried to exchange the mobile phase salt of the traditional HIC methods, from the hazardous ammonium sulphate to ecofriendly salts like sodium citrate or potassium phosphate in the separation of pVAX1GFP (3697 bp), from bacterial lysates [70]. The mobile phases used are phenyl agarose adsorbent (HIC support) and mercaptopyrimidine-agarose adsorbents (thiophilic aromatic chromatography support). The method was adapted to be performed directly on the bacterial lysate without any preliminary preparatory steps. The elution system containing 1.5 M sodium citrate with mercaptopyrimidine-agarose adsorbent recovered 91.1% of pDNA [70]. Nevertheless, this elution system obtained very low pDNA purity (13.4%), suggesting it to be a preliminary purification step because of its high recovery. While in a single step, 2 M potassium phosphate elution system with mercaptopyrimidine-agarose column as well, recovered 68.5% of pDNA with purity of 98.8%. Despite the low recovery, the exalted purity of the obtained pDNA is enough to meet the international vaccine purity recommendations. Moreover, the cleanness, cost effectiveness and quickness of this method make it valid for application as a single-step pDNA purification method. However, the separation between the pDNA isoforms is still invalid using this method [70]. In order to provide a new cost effective single step HIC for purification of sc pDNA isoform, Bo's team conducted a research [71]. The research team utilized Octyl Sepharose 4 Fast Flow adsorbent as column packing material. The mobile phase used was ascending concentration of ammonium sulphate (from 2 to 3 M) in 40 mM Tris/HCl, at pH 8 using pCDNA3.1-*GFP* clarified lysate as a sample. The elution was monitored using agarose gel electrophoresis and anion exchange HPLC. Purification of sc pDNA was performed with 98% ± 1.2% purity and 75% recovery. Bo et al.'s method is considered quick and simple tool for purification of sc pDNA directly from bacterial lysates; nevertheless, the use of ammonium sulphate may limit this method because of its harmful effects [71].

In the same year, Limonta et al. tried to improve the productivity and reduce the cost of production of HCV pDNA vaccine (pIDKE2) through simulating the large scale purification in a laboratory scale [72]. The researchers used a newly design manufacturing technology called Convective Interaction Media (CIM) technology on a lab scale. CIM C4 HLD monolithic column was utilized and the sample was washed by 25 mmoL/L Tris-HCl (loading buffer) and then eluted will 25 mmoL/L Tris-HCl, 10 mmoL/L EDTA pH 7.0. CIM technology was found to be superior to the traditional beads media in terms of cost (39% decrease in the cost), purity (94%) and recovery (80–90%), see Fig. 7 [72].

In 2014, Rocha et al. designed a study to evaluate the ability of gellan gum (polysaccharide polymer) in separation of pDNA by HIC on an analytical scale [73]. The stationary phase was prepared by adding 2.5% (w/v) gellan and 90 mM CaCl2 to 5% (v/v) DMF, and then left at 90 °C for 14 h. Separation trials of the sc isoform of pVAX mixed with RNA sample were implemented using a two-step elution. The first step; 0.2 M ammonium sulphate in MES buffer and calcium chloride, and the second step; the salt was removed from the elution system [73]. The resulted data showed that the pDNA molecules retained at the first step in the column while RNA and other impurities were eluted. While in the second elution step, all the retained pDNA were liberated, as shown in Fig. 8. Thus, gellan gum can be used as HIC stationary phase. However, more trials are needed to check the feasibility of gellan gum in purification of sc pDNA from lysates [73].

In a novel approach, Üzek et al. used magnetic nanoparticles in purification of pDNA [74]. Emulsion polymerization technique was used to produce magnetic nanoparticles and different elution system conditions were implemented. The best separation took place with elution system of an acetate buffer (pH: 5.5) consisting of 1.0 M Na_2SO_4 at 40 °C and magnetic nanoparticles of size 52.0 nm and polydispersity index of 0.205 as stationary phase. The highest adsorption capacity of the nanoparticles measured was 211.3 mg/g. This method succeeded in separation of oc pDNA from other isoforms and impurities [74]. Although, the oc pDNA is not beneficial for using in vaccines, this method proved the feasibility of using magnetic nanoparticles in pDNA purification because of their high adsorption capacity to pDNA [74]. In 2015, Pyridine was immobilized onto methacrylate monolith to purely separate sc pEGFP-N1. Pyridine monolith was compared to C4HLD monolith using descending ammonium sulphate as an elution system to find that both have the same dynamic binding capacity and recovery and the same ability of separating impurities. However, pyridine-modified monolith separated sc pDNA with better purity (98%) than C4HLD (95%) [75].

3.6. Anion exchange chromatography (AEC)

The Anion exchange chromatography AEC is employed in purification of pDNA using positively charged stationary phases, taking the advantage of the negatively charged phosphate groups of pDNA. Although the lysate contains different topologies which may have the similar overall charge and molecular weight, the differences of the conformations of the isoforms change the distribution of the charge densities [15]. Thus, positively charged stationary phases interact locally with the opposite charges of pDNA isoforms.

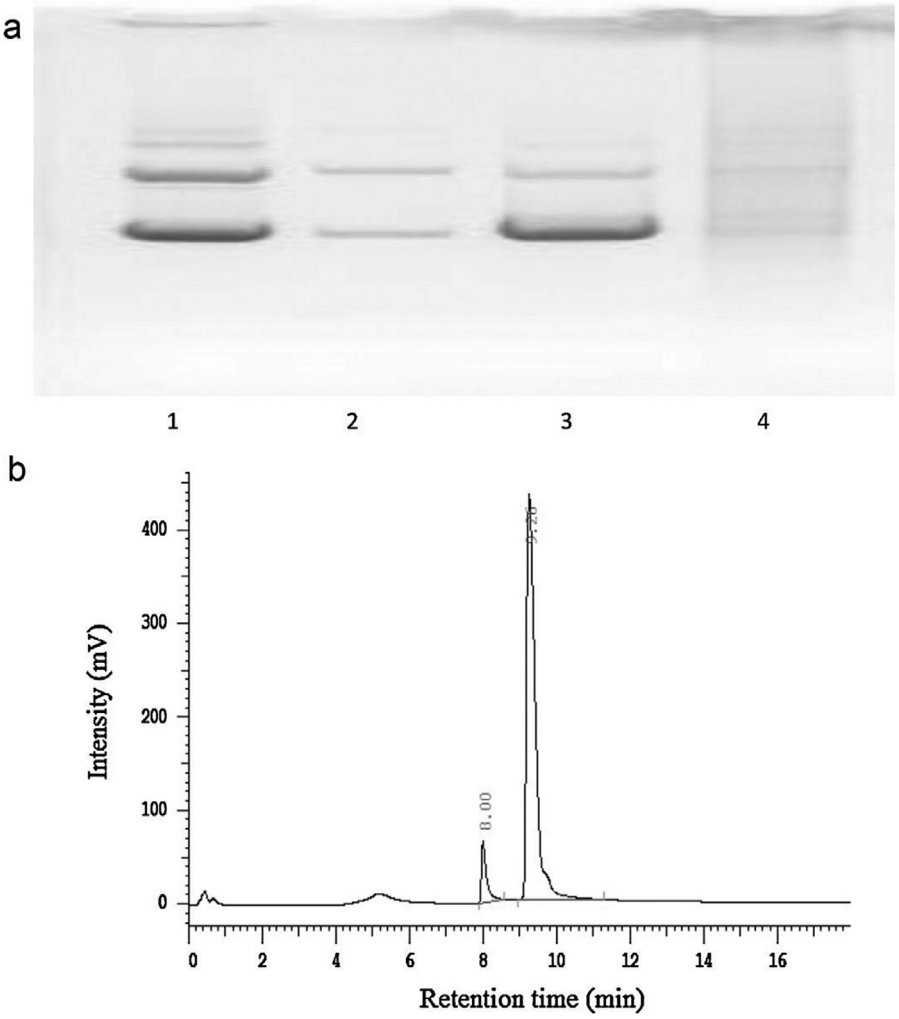

Fig. 7. Recovery of pIDKE2 plasmid using CIM C4 HLD matrix. Collected fractions were analysed by agarose 0.8% electrophoresis, (a) (1) initial sample, (2) not bound fraction, (3) elution fraction, (4) regeneration; (b) chromatographic behavior of elution fraction analysed on CIMacTMpDNA-0.3 analytical column which contains 94%purity of pIDKE2 [72].

Therefore, by elevating the salt concentration, the pDNA isoforms will be separated according to the chain length of the molecules. Longer chain length has more phosphate groups and then higher in charge density [15]. Therefore, the supercoiling of the sc isoform boosts the charge density over that of oc isoform. Accordingly, the sc isoform tightly interacts with the positively charged stationary phase ligand, allowing the sc isoform to elute after the oc isoform [76]. Nevertheless, separation of linear pDNA is not just dependent on the charge density, but also on its physicochemical characteristics. For instance, small DNA fragments may take a coil shape in solutions, which increase its elasticity and enhance its separation from the sc and oc isoforms. Moreover, other interactions like, dispersive forces, hydrogen bonding, dipole-dipole attraction and solvophobic repulsion can also affect the separation by AEC. However, the non-specific binding of the anion exchange stationary phases, affects the pDNA selectivity from other lysate components. The need for appropriate purity of the obtained pDNA may require the use of complementary purification steps like size exclusion chromatography or agarose gel electrophoresis [77,78].

In 2011, Zhong et al. aimed at removal of RNA impurities from pDNA sample without using RNase by firstly, precipitation of pDNA takes place with isopropanol as a pre-purification step [79]. Secondly, anion exchange membrane chromatography was implemented to get rid of the RNA impurities. The stationary phase is an anion exchange membrane incorporated with 3-acrylamidopropyl-trimethylammonium chloride packed in stainless steel membrane disk holder. The elution system is a gradient increase in sodium chloride concentration from 50 mM NaCl in Tris-HCl to 1 M NaCl at pH 8. The recovery of pDNA is 84%. The method is rapid, cheap and efficient; however, it is not convenient for separation of pDNA isoforms [79]. Aiming at purification of large pDNA molecules (25 kbp), Ongkudon et al. innovated a new conical monolith column employed in one step for this purpose, by reducing the wall channel size of monolith column [42]. The whole sample was diluted with buffer A (25 mM Tris—HCl, 2 mMEDTA, pH 7) and then washed by eluting buffer A: buffer B (25 mM Tris—HCl, 2 mM EDTA, 1.0 M NaCl, pH 7) (8:2) to remove the impurities and finally elution was done by mixing buffer A with buffer B to elute the pDNA. The mild conditions of the elution system allowed the researchers to separate large size pDNA (25 kbp) in a reasonable purity and recovery as shown in Fig. 9. Such large pDNA molecules could not be separated in a good yield in drastic methods like affinity chromatographic methods. [42].

In 2015, Bicho et al. employed ion exchange chromatography for purification pDNA influenza vaccine. Ethylenediamine (EDA) was used as a ligand immobilized into monolithic discs with

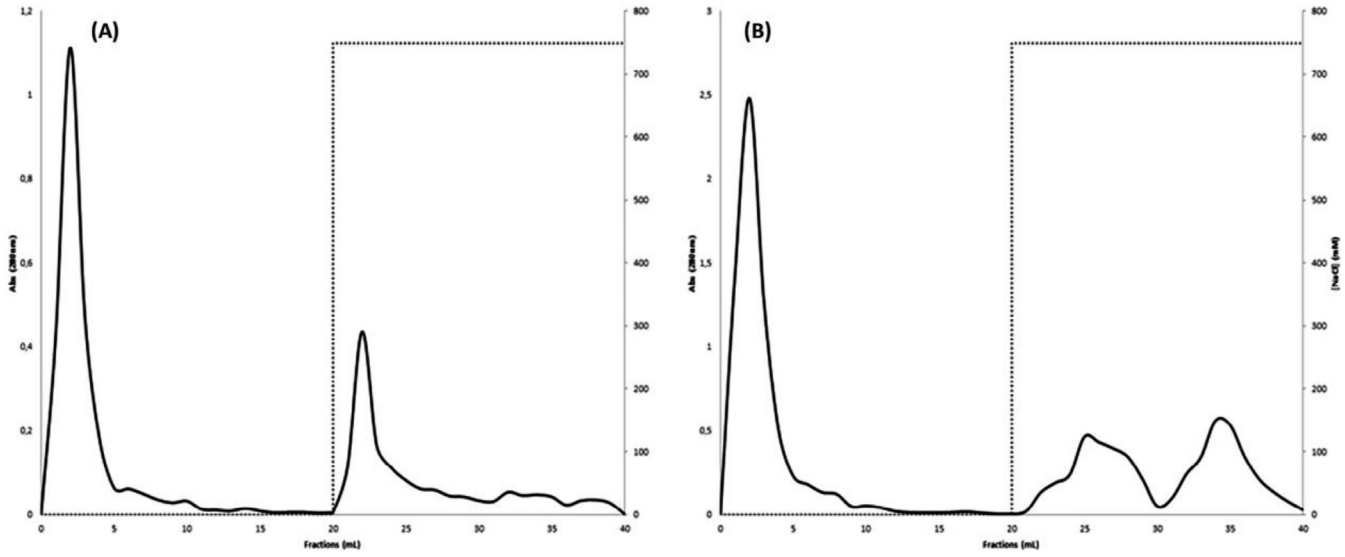

Fig. 8. Elution profiles obtained to the nucleic acid samples. The binding step was made in MES buffer (10 mM, pH 6.2) with CaCl2 (90 mM) and (NH4)2SO4 (2.5 M) and the elution step was made with MES buffer (10 mM, pH 6.2) with CaCl2 (90 mM). Black line and dashed lines represent the absorbance at 280 nm of the fractions and salt concentration, respectively. (A) Obtained profile to the RNA sample. (B) Obtained profile to sc pDNA sample [73].

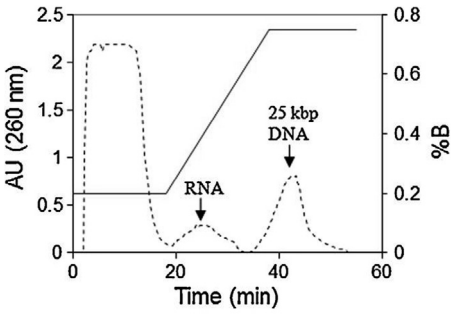

Fig. 9. Anion exchange chromatography of 25 kbp DNA using diethylamine-functionalised polymethacrylate conical monolith. Buffer A (25 mM Tris–HCl, 2 mMEDTA, pH 7). Buffer B (25 mM Tris–HCl, 2 mM EDTA, 1.0 M NaCl, pH 7). Flow rate:1.0 mL/min; Sample: 1 mL of clarified alkaline lysed cell lysate. Dashed line: AU(260 nm); solid line: %B [42].

poly(glycidyl methacrylatecoethylene dimethacrylate) [80]. The sample was pre-purified with Maxi Kit from Qiagen and then loaded into the discs. Different salt concentrations were implemented and the elution was monitored by UV at 260 nm. The separated fractions were analysed using agarose gel electrophoresis and HPLC using CIMac™ pDNA analytical column. It was found that the best separation takes place by gradient increase in NaCl from 1.65 M to 1.85 M in 50 mM phosphate buffer at pH 7.5. The research group explained this on the fact that sc pDNA is negatively charged so it interacts with EDA. Low concentration of the salt (1.65 M) decreases the ionic strength of the mobile phase which keeps the pDNA particles strongly attached to EDA and allows the non-pDNA particles like oc plasmid, RNA molecules, gDNA, proteins and endotoxins to separate [80]. By increasing the salt concentration to 1.85 M, the ionic strength increases allowing the strongly bound pDNA to liberate. The pDNA influenza vaccine was obtained with 97.1% purity and 47% yield. This method is ecofriendly; however, the yield obtained is not satisfactory [80]. Recently, Frenco-Medrano et al. carried out a comparative study between different column packing materials of AEC using Mustang Q membranes and perfusive beads packed columns in separation of the leishmania pDNA vaccine [81]. AEC membrane and perfusion bead purifications were performed using 0.5 × 5 cm (0.08 mL packed volume) column packed with Mustang Q membranes -which contains quaternary amines immobilized on its pores- or Poros 50 HQ beads -which contains quaternized polyethyleneimine group- respectively [81]. The columns were equilibrated with 0.5 M NaCl in TE buffer. The mobile phase used is NaCl in TE buffer where the salt concentration increases gradually via 3 steps. The first one is from 0.6 to 0.725 M NaCl, then from 0.725 to 1.1 M NaCl, and the last step concentration is 2 M to restore the column. The analysis of elution at AEC membranes proved that the pDNA was adsorbed on the membranes and retained and was not affected by the first and the second elution steps. Increasing the ionic strength of the elution system at the third step let the pDNA particles to exit from the column at an acceptable yield (88%) [81]. Comparing to AEC perfusion bead system, small pDNA particles can pass through the pores of the perfusion beads and the adsorption on the beads is not enough just to retain them. Part of the pDNA molecules exits the column during the first two steps of elution, so the yield of the obtained pDNA declines to 63% [81].

3.7. Multimodal chromatography (MMC)

The need of a single chromatographic procedure that exerts more than one interaction with the samples at the same time, led to the invention of multimodal or mixed mode chromatography (MMC). The MMC ligands have more than one active site that can exhibit more than one type of interactions at the same conditions [82]. For example, ligands possess a hydrophobic moiety and an ionic moiety can be used in multimodal chromatography (MMC) to exhibit multiple non-covalent interactions with the intended molecules. This advantage allowed the scientist to use MMC in various purification applications of macromolecules and biomolecules including pDNA [22,83–85]. The recent studies revealed different multimodal ligands like histamine [61] and agmatine [62] for purification of pDNA. Moreover, the MMC allows the investigation group to use single chromatographic interaction as well,

depending on the mobile phase pH as shown in Fig. 10 [82]. For instance, at low pH, the ionic moiety becomes protonated and charged, so ionic chromatography takes place, thus, an anionic elution system like NaCl solution should be used. At basic pH, the ionic moiety becomes uncharged and the hydrophobic moiety's effect dominates, hence, hydrophobic chromatography takes place, and the elution system used should be hydrophobic like ammonium sulphate solution [86].

Histamine was found to have multimodal properties as it shows hydrophobic properties (imidazole ring), and ion-exchange character (amino group) [61]. Histamine's multimodal properties paid the attention of Cernigoj and their co-workers, to be incorporated into MMC as a ligand for separation of pJ plasmid (5.17 kbp) isoforms from clarified *E.coli* strain HMS174 (DE3) lysate [61]. The ligand (histamine) was immobilized onto monolithic CIM CDI column to take advantage of the promising mass transfer of monolith column. The preliminary studies proved that, histamine demonstrated reasonable dynamic binding capacity to pDNA which may be related to the high base exposure and abundance of active sites of histamine which allows it to interact with pDNA. The separation of the sc pDNA isoform was done through two steps using different elution systems on the same column. In the first step, an elution system of ascending gradient of NaCl at acidic buffer was employed to reveal the anion exchange properties of histamine ligand. In this step, the separation of pDNA from other lysate contaminants took place with 90% yield. After that, the obtained fractions was repurified to separate the sc pDNA from oc pDNA using 3 M $(NH_4)_2SO_4$ at descending pH gradient as a mobile phase on the same column to obtain the sc pDNA isoform with 97% yield and 98% purity [61]. The research paper presents efficient procedures for purification of sc pDNA; however, the high concentration of ammonium sulphate in the elution system makes its application costly and ecologically unclean [61]. Moreover, another research group immobilized octylamine, combination of butyl (C4) grafted methacrylate (gBuMA) and diethylaminoethyl (DEAE) onto methacrylate monoliths aiming at purification of pDNA to compare their purification efficiency to that of butyl (C4) and DEAE methacrylate monoliths [87]. The dual interactive abilities of the octylamine and (gBuMA) and (DEAE) ligands are referred to the existence of its hydrophobic chain (like octyl, butyl, ethyl groups) or by its ionic exchange moiety (like amino groups). Investigations proved that octylamine and gBuMA + DEAE monoliths have the best results in terms of binding capacity under both hydrophobic and ion-exchange conditions. Therefore, the experiment was focused on these two columns. The elution system was employed in 4 steps; starts with 0.6 M NaCl in 10 mM EDTA, 50 mM Tris-HCl, then 3 M ammonium sulphate in 10 mM EDTA, 50 mM Tris-HCl, then 3 M ammonium sulphate with 1 M NaCl in 10 mM EDTA, 50 mM Tris-HCl, and finally 1 M NaCl in 10 mM EDTA, 50 mM Tris-HCl [87]. The obtained pDNA showed an acceptable purity with removal of around 99% of the impurities, with about 80% recovery. When it is compared to purification by HIC then IXE, MMC found to be simpler and easier as its exempted in one step; however, the loading sample used in the last one is slightly lower. Both OA and gBuMA + DEAE monoliths could not separate oc and sc isoforms; however, gBuMA + DEAE monolith showed promising data that may need further optimizations. Moreover, the high concentration of ammonium sulphate (3 M) in the elution system may contribute to ecological threats [87]. In 2014, Matos et al. used a novel multimodal resin called “Capto Adhere resin” immobilized with N-benzyl ethanolamine as a stationary phase to separate pUC18 (2.686 Kbp) from *E.coli* extract [88]. Capto Adhere resin was found to undergo ionic and hydrophobic interactions and can bind strongly to nucleic acids with adequate dynamic binding capacity (60 μg/mL). The mobile phase used was gradient increase in NaCl concentration (from 1 M to 2 M) in 10 mM Tris-HCl (pH 8.0), to yield highly pure pDNA with high yield. The researchers tried to separate the two isoforms of pDNA using a linear NaCl gradient; however, the separation was partial and the sc pDNA purity is low (70%) [88].

In 2016, Silva-Santos et al. continues Matos et al. [88] procedures to provide a feasible method for separation of pDNA isoforms (sc pDNA and oc pDNA) from a pDNA extract consists of 45.8% sc to 54.2% oc isoforms [22]. The research team used Capto adhere resin as well; however, this time the Capto adhere resin was immobilized with N-benzyl-N-methyl ethanolamine ligand. The N-benzyl-N-methyl ethanolamine consists of three different interaction sites; the amine group and the hydroxyl group undergo ionic interactions, and the benzyl group undergoes hydrophobic interaction. Two mobile phases were employed; mobile phase (A) is low-salt buffer (A: 10 mM Tris-HCl and 1 mM EDTA-TE, pH 8) and mobile phase (B) is high-salt buffer (B: 2MNaCl in TE, pH 8) with gradient increase in NaCl concentration. The separation took place basing on the fact that the superior charge density of the two topoisomers (oc and sc) might allow them to interact with the charged amino group of the ligand [22]. However, the “torsional strain” of sc pDNA induces the interaction with the hydrophobic

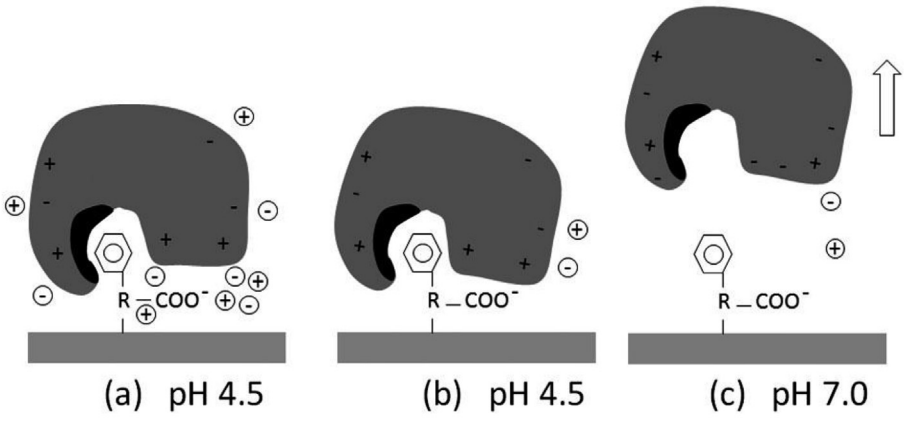

Fig. 10. Multimodal chromatographic principle. The binding and elution strategy of a target protein to a chromatographic medium containing a ligand with both hydrophobic and charged groups (adapted from Ref. [52]). (A) At pH 4.5 a target protein in a mobile phase with a high salt concentration is bound to the hydrophobic part of the ligand. (B) Upon decreasing the salt concentration, but still maintaining a low pH, the electrostatic part of binding becomes the dominating force. (C) With a pH step increase to 7.0, the protein will have the same charge as the ligand and be repelled by it and is therefore eluted [82].

part of the ligand as well, which retains the sc isoform for longer time than the oc isoform. The oc and sc isoforms were separated with purity of 91.8% and 92.2%, respectively. This method can be a complementary method for Matos et al. [88] work, which failed to separate the two isoforms. However, the feasibility of application of Silva-Santos et al. directly onto the bacterial lysates is still unproven. Moreover, the obtained pDNA purity doesn't comply to the pharmaceutical recommendations [22]. In 2016, Bicho et al. performed a research aiming at studying the versatility of agmatine as a multimodal ligand for purification of influenza sc isoform of plasmid NTC7482-41H-VA2HA vaccine from *E.coli* lysate [86]. Agmatine can act as a multimodal ligand, since it has butyl group (hydrophobic moiety) and amino group (ionizable group). Agmatine was first immobilized into CDI monolith column. In order to purify sc pDNA, the investigators tried two strategies of elution. The first one is gradual descending of ammonium sulphate from 3 M to 0 M at 50 mM phosphate buffer at pH 9.6, and the second is gradual ascending of sodium chloride from 1.28 to 2 M at 50 mM phosphate buffer at pH 8. The Separation of sc pDNA was successfully done with 98.3% purity and 51.8% recovery with ammonium sulphate elution system. While separation of pDNA took place with 99.6% purity and 45.3% recovery with sodium chloride elution system [86]. Bicho et al. and their team provided the literature with one-step purification method of sc isoform; however, the procedures require elevated salt concentrations which make it costly and ecologically unsafe. Moreover, the recovery of sc isoform is low [86]. Recently, Silva-Santos et al. [89] tried to optimize their previous method [22] to isolate sc pDNA using the same stationary phase (CaptoTM adhere) immobilized with N- benzyl-N-methyl ethanolamine. This time the sample was prepurified by precipitation with isopropanol then precipitation with ammonium acetate and polyethylene glycol (PEG). The obtained fractions were purified using CaptoTM adhere to isolate sc pDNA. The elution system used was a gradual increase of NaCl concentration in 10 mM Tris-HCl, 1 mM EDTA, pH 8 buffer. At the beginning, a concentration of 830 mM NaCl in the buffer solution was applied as a mobile phase to elute oc pDNA. Then, the NaCl concentration was elevated to 920 mM to liberate sc pDNA. Finally, 2 M NaCl was applied to remove RNA fractions [89]. The method could achieve baseline separation of the three components, indicating a good separation. In order to measure the reproducibility of the method, different sized pDNA molecules [pVAX1-GFP (3696 bp), pUC18 (2687 bp) and pCEP4 (10,410 bp)] were used. The method achieved reproducible results with baseline separation of the pDNA isoforms of the three different pDNA molecules. The researchers tried to purify the pDNA molecules directly after isopropanol precipitation, bypassing the ammonium acetate and PEG precipitation; however, the protein content was found to be higher in the sc fraction which affects the lifetime of the resin [89]. The method is ecofriendly and cost effective as the researchers used low concentrations of NaCl and avoided using ammonium sulphate. Moreover, the yield of the sc and oc pDNA from the multimodal chromatography step is 83%, with final chromatographic purification process yielded sc-rich fractions (>90%) that contains low levels of gDNA and protein impurities. However; the whole purification process could yield only 47% pDNA (oc and sc isoforms) [89].

3.8. Sample displacement chromatography (SDC)

Displacement chromatography is a chromatographic method where the sample is firstly introduced to the column and then displaced by a stronger sorbed solute [90]. Thus, the sample components are separated into successive rectangular zones, allowing the separation of the sample ingredients in high purity. Displacement chromatography columns are usually used as preparative columns as they offer a reliable method to obtain the desired component with high concentration, purity and yield specially with biomolecules [90]. Separation in displacement chromatography depends on the availability of unoccupied active sites of the stationary phase molecules. Separation takes place through three stages; loading stage, displacement stage and regeneration stage. In the loading stage, the biomolecules are introduced into the column to occupy all the stationary phase's active sites and equilibrium takes place [91]. The next stage is the displacement stage, where a solute (displacer) of a higher affinity than the biomolecule is introduced in order to compete with the biomolecule on the active sites of the stationary phase. Thus, the displacer expels the biomolecules to the mobile phase in a high concentration and high purity. The last stage is the regeneration stage, where the displacer is removed by means of washing with organic solvent, large changes in the pH or chemical reaction [91].

In 2015, Cernigoj et al. purified sc pDNA by SDC as a novel technique [92]. In this technique sc pDNA isoform is the displacer of oc and linear pDNA. CIM monolith was used as stationary phase because of its sufficient mass transfer efficiency. The researchers compared the separation efficacy by different CIM monolith columns loaded with different molecules of different hydrophobicities (CIM C4 HLD, CIM-histamine and CIM-pyridine) as illustrated in Fig. 11 [92]. It was concluded that the efficiency of sample displacement depends on the selectivity of the column of a single isoform, column efficiency and concentration of ammonium sulphate in the loading buffer. The method can be a valuable approach for preparative scale because it needs lower concentrations of ammonium sulphate and the obtained pDNA showed acceptable purity [92].

3.9. Miscellaneous chromatographic methods

As was stated in our 2013 review, purification can be done by single or multiple chromatographic steps [15]. Size exclusion chromatography is still usually employed as a polishing step followed other chromatographic procedures. In 2013, Sun et al.

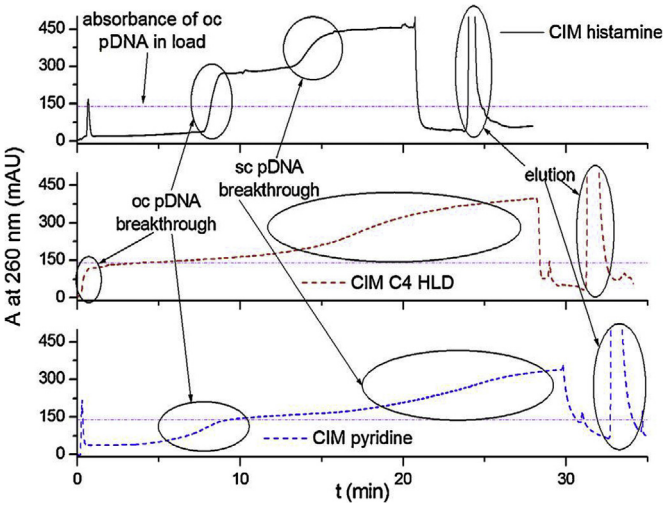

Fig. 11. Loading of a mixture of oc and sc pDNA (pEGFP) with sc pDNA homogeneityof 80% on different ClMac columns in buffers containing specific concentrations ofAS.-Loading buffer: 50 mM TRIS, 10 mM EDTA, different concentrations of ammoniumsulfate (AS), pH 7.4. Elution buffer: 50 mM TRIS, 10 mM EDTA, pH 7.4; Q (volumetricflow rate) = 0.5 mL/min, _ = 260 nm.Pyridine column: _ (oc pDNA) = 3.5 _g/mL, _ (sc pDNA) = 15 _g/mL, c (AS) = 2.0 M.C4 HLD column: _ (oc pDNA) = 4.7 _g/mL, _ (sc pDNA) = 19.8 _g/mL, c (AS) = 1.7 M.Histamine column: _ (oc pDNA) = 4.6 _g/mL, _ (sc pDNA) = 19.0 _g/mL, c(AS) = 2.1 M [92].

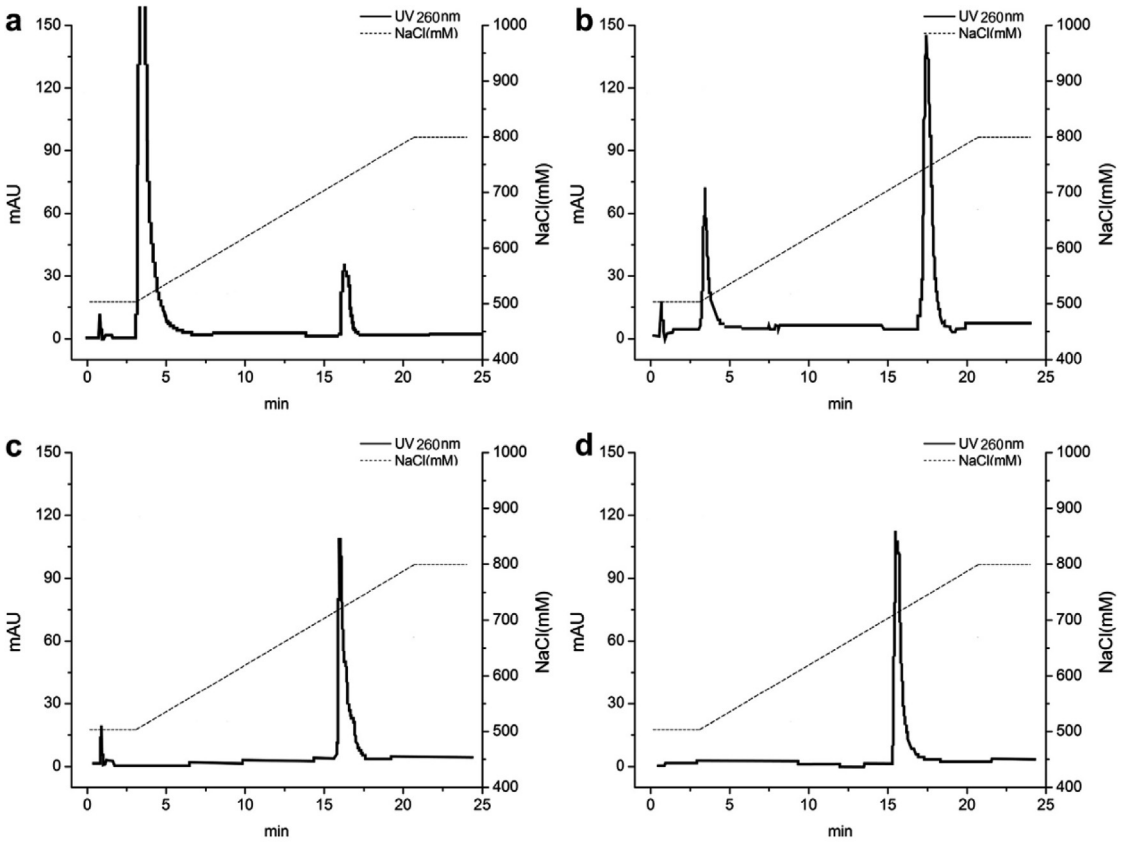

Fig. 12. Mini Q analysis of plasmid DNA containing solutions collected throughout the purification process: (a) clarified lysate, (b) TFF retentate, (c) IEC (Q Sepharose XL) peak II and (d) HIC (Phenyl Sepharose 6 FF) peak I. Using the Mini Q analytical column, plasmid DNA was eluted with a NaCl gradient to 1.0 M in 50 mM sodium phosphate buffer at pH 7.0. The dilution factors of the samples (100 mL) injected were 1:10, 1:100, 1:100 and 1:100, respectively. UV absorbance at 260 nm was used to monitor the chromatography runs [23].

presented a large scale method of pDNA purification by tangential flow filtration, and multistep chromatography using AEC, HIC then size exclusion chromatography [23]. Tangential flow filtration of lysate was to eliminate RNA by pressurizing the lysate at 10–15 psi through Centrasette TFF cassette with a polyether sulfone (PES) membrane and then dialysis against 0.5 M KAc, pH 5.5. The second step is ion exchange chromatography using Q Sepharose XL resin as stationary phase and the elution system was processed via two steps, the first one is 0.6 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 to remove the remaining RNA and the second step in 1.0 M NaCl at the same buffer to elute the pDNA. The eluted solution was concentrated and loaded onto hydrophobic interaction column (Phenyl Sepharose 6 FF). The elution was achieved by decreasing concentration of ammonium sulphate from 2 M to 0 M at 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 [23]. The third purification step used was size exclusion chromatography by passing the last eluted sample through column packed with 5.5 L Sepharose 6 FF resin with elution system of 20 mM PBS, pH 7.2. The last fraction was sterilized, concentrated and analysed using agar gel electrophoresis. The product resulted was pure enough to meet the pharmaceutical grade requirements with minimum amount of impurities. A two kilograms of bacterial cell paste yielded 800 mg of pDNA with overall yield 48% with nearly 100% purity. The Mini Q analysis of the four steps is shown at Fig. 12 [23].

4. Conclusion

The purification step is the key step in pDNA vaccines production. The purity of the pDNA vaccine can affect its efficacy and stability. Thus, the regulatory bodies oblige the manufacturers to prepare the pDNA vaccines in certain purity that can achieve the best efficacy and stability. The instability of the pDNA towards temperature, pH and mobile phase salt concentration is the main hurdle facing researchers during their purification trials. Over the last five years, many research teams exerted their efforts on purification of pDNA especially the sc isoform using chromatography. The last few years witnessed a change in scholars' heed in purification of pDNA vaccines. Size exclusion and reversed phase chromatography are no longer used as a single purification step. Both are generally employed mixed with other chromatographic modalities. In comparing to our review in 2013 [15], there is an increasing interest in affinity chromatography for the purification of pDNA vaccines in the last five years. This may be attributed to the high selectivity and specificity of affinity chromatography [46]. However, the deteriorating effect of salts and pH used limits its use in separation of large size pDNAs. Furthermore, AEC is still of concern of many researchers in isolation of pDNA; however, it is no longer the predominant chromatographic method as was viewed previously in 2013. In addition, the hydrophobic properties of pDNA allowed the scholars to enrich the literature with separation methods via HIC. Moreover, the promising chromatographic modality; MMC, started to attract many research teams as a purification tool of pDNA during the last few years which was not common before 2013. On the other hand, some chromatographic techniques have declined; for example, tentacle chromatography. The latter is one of the techniques that has not been well explored. Furthermore, expanded bed chromatography did not attract research groups in the last five years due to its limited loading capacity [15].

In contrary, the introduction of SDC in purification of pDNA is yet considered a new promising approach. Although the preliminary studies showed an acceptable degree of pDNA purity that qualifies this technique to be used in the industrial scale, purification of pDNA by SDC technique is still not well investigated.

The advantages of Convective Interaction Media (CIM) monolith stationary phases, including low backpressure and quick separation of macromolecules, qualified them to be used effectively in separation of pDNA. CIM monolith stationary phases enabled them to be implemented in different chromatographic modalities including affinity, hydrophobic interaction, anion exchange and multimodal chromatographies. As it was predicted in our last review, methacrylate monolith has proved its efficacy in purification of pDNA due to its outstanding productivity. The low back pressure of the methacrylate monolith columns, allowed the researchers to use the same columns in the small and the large scales. This assists the investigators to explore the column's industrial behavior in the laboratory giving a deep insight on their industrial application.

Although scientists have achieved a notable success in the field of purification of pDNA vaccines, more investigations are still needed to be carried out. The clarification and pre-purification steps may affect the yield obtained after purification. Thus, the need of a single chromatographic step that allows the separation of pDNA in an adequate purity and yield without a clarification or pre-purification step is still not well investigated. Moreover, the environmental considerations should be taken into account while choosing the elution salt. The use of hazardous salts should be avoided and replaced with ecofriendly cheap salts like sodium chloride. Furthermore, more efforts should be exerted for separation of labile large pDNA molecules that can be affected by pH and salt concentration of the mobile phases.

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